BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 20(3), 340-345 (2012)

Sarsasapogenin Increases Melanin Synthesis via Induction of Tyrosinase and Microphthalmia-Associated Transcription Factor Expression in Melan-a Cells

Eunjung Moon[1], Ae-Jung Kim[2] and Sun Yeou Kim[3,*]

[1]Graduate School of East-West Medical Science, Kyung Hee University Global Campus, Yongin 446-701,
[2]Graduate School of Alternative Medicine, Kyonggi University, Seoul 120-702,
[3]College of Pharmacy, Gachon University, Incheon 406-799, Republic of Korea

Abstract

Sarsasapogenin (SAR) is a steroidal sapogenin that is used as starting material for the industrial synthesis of steroids. It has various pharmacological benefits, such as antitumor and antidepressant activities. Since its effect on melanin biosynthesis has not been reported, we used murine melanocyte melan-a cells to investigate whether SAR influences melanogenesis. In this study, SAR significantly increased the melanin content of the melan-a cells from 1 to 10 μM. Based on an enzymatic activity assay using melan-a cell lysate, SAR had no effect on tyrosinase and DOPAchrome tautomerase activities. It also did not affect the protein expression of tyrosinase-related protein 1 and DOPAchrome tautomerase. However, protein levels of tyrosinase and microphthalmia-associated transcription factor were strongly stimulated by treatment with SAR. Therefore, our reports suggest that SAR treatment may induce melanogenesis through the stimulation of tyrosinase and microphthalmia-associated transcription factor expression in melan-a cells.

Key Words: Sarsasapogenin, Melanogenesis, Hyperpigmentation, Tyrosinase, Microphthalmia-associated transcription factor

INTRODUCTION

The biosynthesis of melanin pigment, or melanogenesis, is a major defense mechanism against ultraviolet radiation (UVR). Melanin is produced in specialized membrane-bound organelles (melanosomes) in melanocytes of the skin (Riley, 1997), and the melanosomes, which contain the melanin pigments, are then transferred to neighboring keratinocytes through the dendrites. The melanin granules accumulate above the nuclei of the keratinocytes and absorb harmful UVR. Therefore, these compounds protect the skin from UVR-induced DNA damage (Kobayashi *et al.*, 1998).

Melanogenesis is associated with several skin disorders, such as vitiligo. Vitiligo is a common disorder characterized by white spots appearing on the skin of the body, and more than 1% of the general population suffers from this disease. Vitiligo is caused by the loss of functional melanocytes (Kovacs, 1998), and its treatment is often difficult because of the lack of effective methods for restoring the pigmentation (Jeon *et al.*, 2007). Therefore, agents that can induce melanogenesis may play important roles in damaged and pathogenic skin. Several studies have reported not only hyperpigmentary factors, including stem cell factor (Grabbe *et al.*, 1994) and basic fibroblast growth factor (Halaban *et al.*, 1987), but also melanogenic inducers, including bovine pituitary extract (Wilkins *et al.*, 1985), 12-O-tetradecanoylphorbol-13-acetate (Krasagakis *et al.*, 1993), bee venom (Jeon *et al.*, 2007), and cAMP elevating agents, such as forskolin, 3-isobutyl-l-methylxan-thine, α-melanocyte stimulating hormone, and glycyrrhizin (Wong and Pawelek, 1975; Halaban *et al.*, 1984; Hunt *et al.*, 1994; Lee *et al.*, 2005). These factors induce melanin biosynthesis in *in vitro* system, though they are rarely used clinically.

Melanin biosynthesis in melanocytes is associated with several melanogenic factors, including tyrosinase, tyrosinase-related protein 1 (TRP-1), DOPAchrome tautomerase (DCT), and microphthalmia-associated transcription factor (MITF). Tyrosinase is the key enzyme in pigment synthesis, being responsible for the first two rate-limiting steps of melanogenesis: (1) the hydrolysis from the metabolic precursor of melanin, L-tyrosine, to L-dihydroxyphenylalanine (L-DOPA) and (2) the oxidation from L-DOPA to dopaquinone (del Marmol and Beermann, 1996). TRP-1 and DCT play important roles in modify-

www.biomolther.org

Open Access **http://dx.doi.org/10.4062/biomolther.2012.20.3.340**

pISSN: 1976-9148 eISSN: 2005-4483

Received May 9, 2012 **Revised** May 18, 2012 **Accepted** May 18, 2012

***Corresponding Author**
E-mail: sunnykim@gachon.ac.kr
Tel: +82-32-899-6411, Fax: +82-32-899-6591

ing the melanin into different types. They are responsible for the production of brownish 5,6-dihydroxyindole-2-carboxylic acid-rich eumelanins. Moreover, TRP-1 plays important roles in the correct trafficking and stabilization of tyrosinase (Hearing, 2000; Toyofuku *et al.*, 2001), and DCT is known to be involved in tyrosinase detoxification (Jimenez-Cervantes *et al.*, 1994). MITF is a transcriptional regulator that plays a key factor in melanin synthesis by activating the gene expression of tyrosinase, TRP-1, and DCT (Bertolotto *et al.*, 1998a; 1998b).

Recently, antimelanogenic activity of diosgenin in B16 melanoma cells was reported (Lee *et al.*, 2007). After confirming that diosgenin also decreased melanin synthesis in a murine melanocyte melan-a cell line, we screened various steroidal compounds that have structural similarity to diosgenin with melan-a cells. We found that sarsasapogenin (SAR) increased melanin production significantly. SAR is a spirostan-3-ol type of steroidal sapogenin found in the rhizome of *Anemarrhena asphodeloides* (Liliaceae). It has been used as starting material for the industrial synthesis of steroids. Its pharmacological activities are known as anticancer (Bao *et al.*, 2007), antidepressant (Ren *et al.*, 2007), improvement of learning ability and memory (Hu *et al.*, 2005), and antioxidant (Ma *et al.*, 2001). The effect of SAR on melanogenesis has not been reported in the literature. Therefore, we confirmed the effect of SAR on melanin synthesis in a murine melanocyte melan-a cell line and investigated the precise mechanism of SAR in this study.

MATERIALS AND METHODS

Materials

RPMI1640 media and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). Penicillin-streptomycin (PS) was purchased from Invitrogen (Grand Island, NY, USA). All other chemicals, including SAR, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of the highest purity grade. Primary and secondary antibodies were purchased from Santa Cruz Biotech (Delaware Ave, CA, USA) and Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture

Murine melanocyte melan-a cells were kindly supplied from Dr. Byeong Gon Lee at the Skin Research Institute, Amore-Pacific Co. (Yongin, Korea). Melan-a cells were maintained in RPMI1640 supplemented with 10% FBS, 1% PS, and 200 nM 12-*O*-tetradecanoylphorbol-13-acetate and incubated at 37°C in a humidified incubator with 5% CO_2.

Melanin contents and cell viability assay

The melan-a cells were seeded with 1×10^5 cells/well in a 24-well plate and incubated for 72 h after being treated with SAR and diosgenin. After 72 h, the melanin contents were measured using a modification of the methods reported by Hosoi *et al.* (1985). In brief, after removing the media, the cells were washed twice with phosphate-buffered saline. Then 1 N sodium hydroxide solution (1 ml) was added to each well to dissolve the melanin contents. The melanin absorbance was measured at 405 nm using a microplate reader.

The percentage of viable cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. After treatment and incubation of the samples for 72 h, as described in the previous paragraph, the medium was removed and 100 μl of 0.1 mg/ml MTT solution was added to each well and incubated at 37°C for 1 h. The MTT solution was then removed and 100 μl dimethyl sulfoxide was added to each well. The formazan formation was measured by absorbance at 570 nm in a microplate reader.

Cell-originated tyrosinase activity assay

Melan-a cells were seeded with approximately 5×10^5 cells in 100-mm culture dishes. When the cells were confluent, they were detached from the dishes and gathered. The cell pellets were disrupted with tyrosinase buffer (1% Triton-X100 and 100 μl/ml phenylmethylsulfonyl fluoride in 80 mM phosphate buffer) via an ultrasonicator in an ice bath. The cells were centrifuged, and the supernatants were used for the enzyme assay. The cell-originated tyrosinase activity assay required 150 μg of protein (Fuller *et al.*, 2000). The tyrosinase activity was determined by measuring the rate of oxidation of L-DOPA (Takahashi and Parsons, 1990). For the DOPA oxidation assay, 40 μl of each sample and 120 μl L-DOPA (8.3 mM in 80 mM phosphate buffer, pH 6.8) were mixed with proteins and incubated in a dark place for 20 min at 37°C. The tyrosinase activity was calculated at 490 nm with a microplate reader. The tyrosinase specific inhibitor, kojic acid, was used as a positive control (Cabanes *et al.*, 1994).

Cell-originated DCT activity assay

Melan-a cells were seeded with approximately 5×10^5 cells in 100-mm culture dishes. When the cells were confluent, they were detached from the dishes and gathered. The cells were then disrupted with a hypotonic medium (80 mM phosphate buffer containing 1% Brij 35, pH 6.8) via an ultrasonicator in an ice bath. After 1 h, the cells were centrifuged and the supernatants were used for the enzyme assay. The cell-originated DCT activity assay used 7 mg/ml of protein (Fuller *et al.*, 2000). DOPAchrome was obtained by mixing cold L-DOPA and silver oxide. L-DOPA (0.5 mg) was dissolved in 1 ml 80 mM phosphate buffer (pH 6.8), and 3 mg silver oxide was dissolved in 1 ml L-DOPA solution. After 3 min of mixing, the mixtures were filtered through a 0.22-μm filter. To remove all impurities from the silver, the filtrate was treated with 30% (v/v) Chelex 100 with gentle agitation. For examination of SAR's inhibitory effect on DCT activity, 20 μl of 1, 5, and 10 μM SAR, 50 μl of 80 mM phosphate buffer (pH 6.8), and 50 μl DOPAchrome substrate were mixed with 25 μl crude DCT. DCT activity was calculated at 340 nm with a microplate reader after 20 min of incubation.

Western blot analysis

To detect the protein levels of tyrosinase, TRP-1, DCT, and MITF, melan-a cells were treated with SAR for 72 h. The cells were then lysed in cold lysis buffer (0.1% sodium dodecyl sulfate, 150 mM sodium chloride, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, 100 μg/ml phenylmethanesulfonylfluoride, 1 μg/ml aprotinin, and phosphatase inhibitor in 50 mM Tris-Cl, pH 8.0). Samples of the resulting protein (40 μg) were separated on 10% acrylamide gels and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% non-fat skim milk in Tris-buffered saline-T and incubated overnight with the primary antibodies at 4°C. The membranes

were then incubated with secondary antibodies for 1 h at room temperature. The membranes were developed using ECL Reagents (Amersham Pharmacia Biotech). Densitometry analysis of the bands was performed with the ImageMaster™ 2D Elite software, version 3.1 (Amersham Pharmacia Biotech).

Statistical analysis

Data were expressed as means ± S.D. from at least three independent experiments. A Student's t-test was used for statistical analyses, and only *p*-values <0.05 were reported as significant. **p*<0.05 and ****p*<0.001 indicate statistically significant differences from the control group.

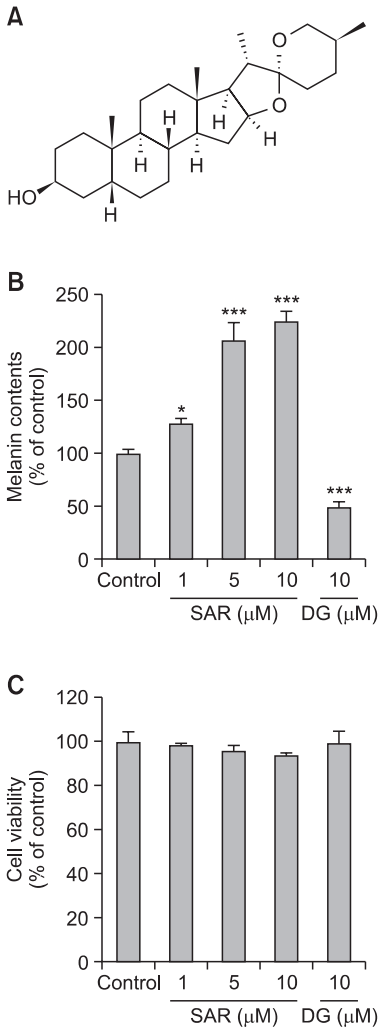

Fig. 1. Effects of SAR on melanin synthesis and cell viability in melan-a cells. (A) The chemical structure of SAR. (B) Melanin contents and (C) cell viability. SAR (1, 5, and 10 μM) and diosgenin (DG, 10 μM) were tested in melan-a cells. Melanin contents and cell viabilities of melan-a cells were tested after 72 h of daily treatment. The results are averages of three independent experiments, and data are expressed as means ± SD. **p*-value <0.05 and ****p*-value <0.001).

RESULTS

Effects of SAR on melanin synthesis and cell viability in melan-a cells

Recently, a study found out that inhibitory activity of diosgenin on melanogenesis in B16 melanoma cells was reported (Lee *et al.*, 2007). We already confirmed that it had reduced melanin contents in melan-a cells. We also screened the effect of various steroidal compounds including diosgenin on melanogenesis in melan-a cells. On the contrary of diosgenin, we found that SAR (Fig. 1A) increased melanin production significantly compared with diosgenin. Therefore, to compare the effects on melanogenic regulation between the two substances, we tried to perform melanin content and cell viability assays after treatment of SAR and diosgenin in melan-a cells for 72 h. As shown in Fig. 1B, 10 μM of diosgenin decreased melanin content. However, SAR significantly stimulated melanogenesis. SAR increased melanin contents by 128.0 ± 6.1%, 207.2 ± 17.0%, and 224.6 ± 10.0% compared to the control at concentrations of 1, 5, and 10 μM, respectively. Both SAR and diosgenin had no influence on cell viability in the same concentration range in melan-a cells (Fig. 1C). Therefore, SAR strongly increased melanin synthesis without cell toxicity in the tested concentrations.

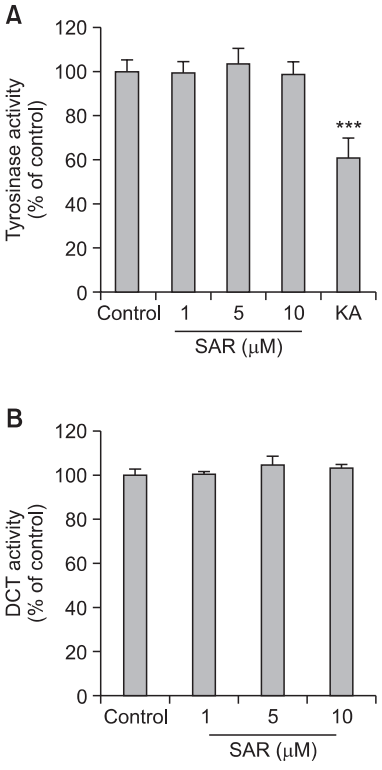

Fig. 2. Effects of SAR on melan-a-originated tyrosinase and DCT activities. (A) Tyrosinase activity. To determine the effect of SAR on tyrosinase activity, 150 μg of proteins from the melan-a cell extracts were reacted with each sample. Kojic acid (KA, 100 μg/ml) was used as a positive control. (B) DCT activity. Protein from the melan-a cell extracts (7 mg/ml) were treated with SAR and DOPAchrome substrate for 20 min. The results are averages of three independent experiments, and data are expressed as means ± SD. ****p*-value <0.001).

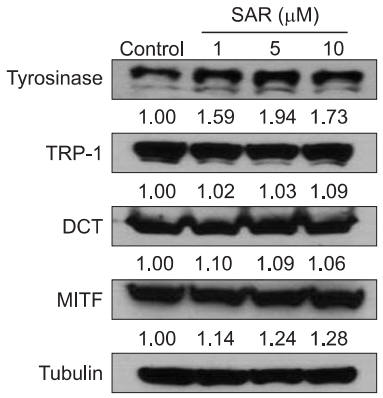

Fig. 3. Effects of SAR on expression of melanogenic proteins in melan-a cells. To determine the protein levels of tyrosinase, TRP-1, DCT, and MIFT, melan-a cells were treated with 1, 5, and 10 μM of SAR for 72 h. Protein cell lysates (40 μg) were used for Western blot analysis. Equal protein loadings were confirmed using anti-tubulin antibody. Densitometry analysis of the bands was performed as described in the methods section. The results are averages of three independent experiments, and data are expressed as means.

Effects of SAR on cell-originated tyrosinase and DCT activities

To confirm the effects of SAR on melanogenic enzyme activity, we performed tyrosinase and DCT activity assays on melan-a cell lysates. As shown in Fig. 2A, SAR had no influence on cell-originated tyrosinase activity. SAR also did not show significant change in DCT activity (Fig. 2B).

Effects of SAR on expression of melanogenic proteins in melan-a cells

To investigate the mechanism of SAR on hyperpigmentation, we determined the protein levels of melanogenesis-regulated proteins including tyrosinase, TRP-1, DCT, and MITF by Western blot analysis after treatment with SAR for 72 h in melan-a cells. As seen in Fig. 3, SAR had no influence on TRP-1 and DCT protein expressions. However, the protein levels of tyrosinase and MITF were increased from 1 to 10 μM by SAR.

DISCUSSION

As the most external portion of the body, the skin often suffers from various environmental stresses, such as UVR. Melanogenesis is considered a protective mechanism against UVR-induced DNA damage (Kobayashi *et al.*, 1998). Therefore, melanin production in the skin is very important to human health. Moreover, depigmenting diseases, such as vitiligo, can lead to depression and similar mood disorders in patients who are stigmatized for their hypopigmentary condition. Recently, we focused on determining the compound that induces melanin production in melanocyte cells. Many previous studies have reported the role of steroids in stimulating melanogenesis. Cholesterol (Schallreuter *et al.*, 2009), vitamin D analogs (Birlea *et al.*, 2009), ovarian hormones, including estradiol and estriol (Maeda *et al.*, 1996), and glucocorticoids, such as dexamethasone (Ito *et al.*, 1991), have been known as hyperpigmentary agents. Following *in vitro* screening of various steroidal compounds from natural resources in melan-a cells, we confirmed the strong effect of SAR on the stimulation of melanin production. SAR is a steroidal sapogenin that has been used as starting material for the industrial synthesis of various steroids. Further study will be needed to investigate the relationship between steroidal structure and regulation of melanogenesis.

Pigmentation may result from the stimulation of melanocyte proliferation, melanogenesis, and migration, or dendritogenesis (Lan *et al.*, 2005; Scott *et al.*, 2006). Among them, this study focused on the materials that can promote melanogenesis. Tyrosinase is the key enzyme in melanin production, initiating a cascade of reactions that convert the precursor of melanogenic pathway, L-tyrosine, to the melanin polymer (del Marmol and Beermann, 1996). MITF is the key transcriptional regulator in melanogenesis. It activates the gene expression of melanogenic enzymes, and it is a nuclear component of the signal transduction pathway (Bertolotto *et al.*, 1998a). It was also shown to bind specifically to the M box and E box motifs and to up-regulate tyrosinase promoter activities (Bertolotto *et al.*, 1998b). Therefore, the stimulation of tyrosinase and MITF levels induces melanogenesis. In this study, SAR increased the expression of tyrosinase and MITF. This indicates that SAR may stimulate melanin production through the increase of tyrosinase and MITF protein levels. Moreover, as shown in Fig. 1C, SAR treatment has no influence on the percentage of viable cells. Therefore, our reports suggest that the increase in melanin levels by SAR may not result from the stimulation of melanocyte proliferation. In this study, we did not examine the effects of SAR on melanocyte migration and dendritogenesis. Further studies will be needed to investigate not only melanogenesis-related signal transduction, such as mitogen-activated protein kinases activation and cyclic AMP, but also the effects of SAR on melanocyte migration and dendritogenesis.

In this study, SAR significantly increased protein level of tyrosinase (Fig. 3). However, Fig. 2A shows that SAR had no influence on cell-originated tyrosinase activity. The inconsistency between the enzyme activity and protein expression level of tyrosinase might have been caused by tyrosinase enzyme activity assay system. Tyrosinase enzymatic activities can be measured in cell system and/or cell-free system (Kim *et al.*, 2005). To investigate the direct effect of SAR on tyrosinase activity, we performed tyrosinase enzymatic activity assay in cell-free system using melan-a cell lysates in this study. Further study is needed to measure the change of tyrosinase activity in melan-a cells after treatment with SAR.

A previous study reported that diosgenin decreased melanogenesis in B16 melanoma cells (Lee *et al.*, 2007). We also confirmed that diosgenin reduced melanin contents in melan-a cells. Although, the structure of SAR (25*S*-5β-spirostan-3β-ol) is similar to diosgenin (25*R*-spirost-5-en-3β-ol), SAR increased melanin production in melan-a cells. The difference of effects seems to be specific to a cis-linkage between rings A and B of the steroid nucleus due to a 5β-H configuration and a (*S*)-configuration at C-25. We suggest that the A/B-cis linkage via a 5β-H configuration may be more important than the (*S*)-configuration at C-25 for various pharmacological activities. Previous studies reported that SAR had anti-diabetic effects, such as reducing blood glucose and reversing weight gain in diabetic animal models; however, diosgenin, with a Δ^5 double bond, had a much lower anti-diabetic activity than did SAR

(Applezweig, 1987). Moreover, SAR had the ability to stop the decline in muscarinic acetylcholine receptors (mAChRs) in animal models of Alzheimer's disease (Hu *et al.*, 2005). However, tigogenin, with a 5α-configuration, showed no effect on mAChRs . Therefore, A/B-cis linkage and 5β-H configuration in spirostane-type steroids may also be related to melanogenic regulation. In further studies, we will investigate the influence of various spirostane-type steroids with 5α- or 5β-configuration on melanogenesis.

In this study, SAR significantly induced melanin synthesis in murine melanocytes melan-a cells. Although SAR did not influence melanogenic enzyme activity, the protein expressions of tyrosinase and MITF were strongly stimulated by treatment with SAR. These results suggest that SAR treatment may induce hyperpigmentation through the increase of tyrosinase and MITF protein expression in melan-a cells. Therefore, this study supports the hypothesis that SAR from the rhizomes of *A. asphodeloides* may be used for the treatment of hypopigmentation-related diseases, such as vitiligo. We are currently working on indicating the molecular mechanisms of SAR for the induction of hyperpigmentation in melanocytes from different origins.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0025362).

REFERENCES

Applezweig. (1987) Treatment of obesity and diabetes using sapogenins. US4680289.

Bao, W., Pan, H., Lu, M., Ni, Y., Zhang, R. and Gong, X. (2007) The apoptotic effect of sarsasapogenin from Anemarrhena asphodeloides on HepG2 human hepatoma cells. *Cell Biol. Int.* **31**, 887-892.

Bertolotto, C., Abbe, P., Hemesath, T. J., Bille, K., Fisher, D. E., Ortonne, J. P. and Ballotti, R. (1998a) Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J. Cell Biol.* **142**, 827-835.

Bertolotto, C., Buscá, R., Abbe, P., Bille, K., Aberdam, E., Ortonne, J. P. and Ballotti, R. (1998b) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol. Cell Biol.* **18**, 694-702.

Birlea, S. A., Costin, G. E. and Norris, D. A. (2009) New insights on therapy with vitamin D analogs targeting the intracellular pathways that control repigmentation in human vitiligo. *Med. Res. Rev.* **29**, 514-546.

Cabanes, J., Chazarra, S. and Garcia-Carmona, F. (1994) Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J. Pharm. Pharmacol.* **46**, 982-985.

del Marmol, V. and Beermann, F. (1996) Tyrosinase and related proteins in mammalian pigmentation. *FEBS Lett.* **381**, 165-168.

Fuller, B. B., Drake, M. A., Spaulding, D. T. and Chaudhry, F. (2000) Downregulation of tyrosinase activity in human melanocyte cell cultures by yohimbine. *J. Invest. Dermatol.* **114**, 268-276.

Grabbe, J., Welker, P., Dippel, E. and Czarnetzki, B. M. (1994) Stem cell factor, a novel cutaneous growth factor for mast cells and melanocytes. *Arch. Dermatol. Res.* **287**, 78-84.

Halaban, R., Ghosh, S. and Baird, A. (1987) bFGF is the putative natural growth factor for human melanocytes. *In Vitro Cell Dev. Biol.* **23**, 47-52.

Halaban, R., Pomerantz, S. H., Marshall, S. and Lerner, A. B. (1984) Tyrosinase activity and abundance in Cloudman melanoma cells. *Arch. Biochem. Biophys.* **230**, 383-387.

Hearing, V. J. (2000) The melanosome: the perfect model for cellular responses to the environment. *Pigment Cell Res.* **13 Suppl 8**, 23-34.

Hosoi, J., Abe, E., Suda, T. and Kuroki, T. (1985) Regulation of melanin synthesis of B16 mouse melanoma cells by 1 alpha, 25-dihydroxyvitamin D3 and retinoic acid. *Cancer Res.* **45**, 1474-1478.

Hu, Y., Xia, Z., Sun, Q., Orsi, A. and Rees, D. (2005) A new approach to the pharmacological regulation of memory: Sarsasapogenin improves memory by elevating the low muscarinic acetylcholine receptor density in brains of memory-deficit rat models. *Brain Res.* **1060**, 26-39.

Hunt, G., Todd, C., Cresswell, J. E. and Thody, A. J. (1994) Alpha-melanocyte stimulating hormone and its analogue Nle4DPhe7 alpha-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. *J. Cell Sci.* **107**, 205-211.

Ito, A., Tanaka, C., Takeuchi, T. and Mishima, Y. (1991) Glucocorticoid stimulates melanogenesis and tyrosinase gene expression in B16 melanoma cells. *Pigment Cell Res.* **4**, 247-251.

Jeon, S., Kim, N. H., Koo, B. S., Lee, H. J. and Lee, A. Y. (2007) Bee venom stimulates human melanocyte proliferation, melanogenesis, dendricity and migration. *Exp. Mol. Med.* **39**, 603-613.

Jiménez-Cervantes, C., Solano, F., Kobayashi, T., Urabe, K., Hearing, V. J., Lozano, J. A. and García-Borrón, J. C. (1994) A new enzymatic function in the melanogenic pathway. The 5,6-dihydroxyindole-2-carboxylic acid oxidase activity of tyrosinase-related protein-1 (TRP1). *J. Biol. Chem.* **269**, 17993-18000.

Kim, D. S., Kim, S. Y., Park, S. H., Choi, Y. G., Kwon, S. B., Kim, M. K., Na, J. I., Youn, S. W. and Park, K. C. (2005) Inhibitory effects of 4-n-butylresorcinol on tyrosinase activity and melanin synthesis. *Biol. Pharm. Bull.* **28**, 2216-2219.

Kobayashi, N., Nakagawa, A., Muramatsu, T., Yamashina, Y., Shirai, T., Hashimoto, M. W., Ishigaki, Y., Ohnishi, T. and Mori, T. (1998) Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. *J. Invest. Dermatol.* **110**, 806-810.

Kovacs, S. O. (1998) Vitiligo. *J. Am. Acad. Dermatol.* **38**, 647-666.

Krasagakis, K., Garbe, C., Krüger-Krasagakes, S. and Orfanos, C. E. (1993) 12-O-tetradecanoylphorbol-13-acetate not only modulates proliferation rates, but also alters antigen expression and LAK-cell susceptibility of normal human melanocytes *in vitro*. *J. Invest. Dermatol.* **100**, 653-659.

Lan, C. C., Chen, G. S., Chiou, M. H., Wu, C. S., Chang, C. H. and Yu, H. S. (2005) FK506 promotes melanocyte and melanoblast growth and creates a favourable milieu for cell migration via keratinocytes: possible mechanisms of how tacrolimus ointment induces repigmentation in patients with vitiligo. *Br. J. Dermatol.* **153**, 498-505.

Lee, J., Jung, E., Park, J., Jung, K., Park, E., Kim, J., Hong, S., Park, J., Park, S., Lee, S. and Park, D. (2005) Glycyrrhizin induces melanogenesis by elevating a cAMP level in b16 melanoma cells. *J. Invest. Dermatol.* **124**, 405-411.

Lee, J., Jung, K., Kim, Y. S. and Park, D. (2007) Diosgenin inhibits melanogenesis through the activation of phosphatidylinositol-3-kinase pathway (PI3K) signaling. *Life Sci.* **81**, 249-254.

Ma, D., Zhang, J., Sugahara, K., Sagara, Y. and Kodama, H. (2001) Effect of sarsasapogenin and its derivatives on the stimulus coupled responses of human neutrophils. *Clin. Chim. Acta.* **314**, 107-112.

Maeda, K., Naganuma, M., Fukuda, M., Matsunaga, J. and Tomita, Y. (1996) Effect of pituitary and ovarian hormones on human melanocytes *in vitro*. *Pigment Cell Res.* **9**, 204-212.

Ren, L. X., Luo, Y. F., Li, X. and Wu, Y. L. (2007) Antidepressant activity of sarsasapogenin from Anemarrhena asphodeloides Bunge (Liliaceae). *Pharmazie* **62**, 78-79.

Riley, P. A. (1997) Melanin. *Int. J. Biochem. Cell Biol.* **29**, 1235-1239.

Schallreuter, K. U., Hasse, S., Rokos, H., Chavan, B., Shalbaf, M., Spencer, J. D. and Wood, J. M. (2009) Cholesterol regulates melanogenesis in human epidermal melanocytes and melanoma cells. *Exp. Dermatol.* **18**, 680-688.

Scott, G. A., Jacobs, S. E. and Pentland, A. P. (2006) sPLA2-X stimulates cutaneous melanocyte dendricity and pigmentation through a lysophosphatidylcholine-dependent mechanism. *J. Invest. Derma-*

tol. **126**, 855-861.

Takahashi, H. and Parsons, P. G. (1990) In vitro phenotypic alteration of human melanoma cells induced by differentiating agents: heterogeneous effects on cellular growth and morphology, enzymatic activity, and antigenic expression. *Pigment Cell Res.* **3**, 223-232.

Toyofuku, K., Wada, I., Valencia, J. C., Kushimoto, T., Ferrans, V. J. and Hearing, V. J. (2001) Oculocutaneous albinism types 1 and 3 are ER retention diseases: mutation of tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins. *FASEB J.* **15**, 2149-2161.

Wilkins, L., Gilchrest, B. A., Szabo, G., Weinstein, R. and Maciag, T. (1985) The stimulation of normal human melanocyte proliferation in vitro by melanocyte growth factor from bovine brain. *J. Cell Physiol.* **122**, 350-361.

Wong, G. and Pawelek, J. (1975) Melanocyte-stimulating hormone promotes activation of pre-existing tyrosinase molecules in Cloudman S91 melanoma cells. *Nature* **255**, 644-646.